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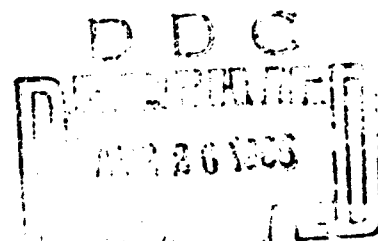
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Disinfection of Aerosolized Pathogenic Fungi on Laboratory Surfaces

I. Tissue Phase

RICHARD H. KRUSE, THERON D. GREEN, RICHARD C. CHAMBERS, AND MARIAN W. JONES

U.S. Army Biological Laboratories, Fort Detrick, Frederick, Maryland

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ABSTRACT

KRUSE, RICHARD H. (U.S. Army Biological Laboratories, Fort Detrick, Frederick, Md.), THERON D. GREEN, RICHARD C. CHAMBERS, AND MARIAN W. JONES. Disinfection of aerosolized pathogenic fungi on laboratory surfaces. I. Tissue phase. *Appl. Microbiol.* 11:436-445. 1963.—The effect of several fungicides on laboratory surfaces contaminated with the tissue phase of aerosolized *Blastomyces dermatitidis*, *Coccidioides immitis*, *Cryptococcus neoformans*, and *Histoplasma capsulatum* was ascertained. A statistical analysis of the data shows the correlation between fungi, surfaces, time, and concentration of disinfectant. All fungicides were effective at established times and concentrations, and the type of contaminated surface affected the fungicidal efficacy. By interpolating plotted graphs, laboratory personnel may determine, with a given fungicide, the concentration and time required to disinfect instruments, pipettes, gloves, bench tops, and floors contaminated with a tissue phase of pathogenic fungi.

Review of the literature has revealed no report on the efficacy of disinfectants for laboratory surfaces contaminated with aerosolized pathogenic fungi such as *Blastomyces*, *Coccidioides*, *Cryptococcus*, and *Histoplasma*. There seems to be no universally accepted test for fungal disinfectants.

The first well-known method of testing germicides employed silk threads impregnated with spores of *Bacillus anthracis* (Koch, 1881). An improvement was made by Rideal and Walker (1903). It and other modifications are still in use today. The phenol coefficient test is recommended, but is not an official test of the Food and Drug Administration. Phillips (1960) stated that the phenol coefficient method is the one most widely used and misused. Reddish (1957) listed the limitations of the phenol coefficient test as: (i) limitation to phenol-like compounds, (ii) variability in resistance to the test culture, (iii) necessity of repeated tests to obtain a final phenol coefficient, and (iv) difficulty in obtaining consistent results. Stedman, Kravitz, and Bell (1954a) regarded much of the controversy as arising from the innate complexity of the disinfecting procedure, resulting from the composition of the surface, the technique used in applying the disinfectant, and the particular organism. Sykes (1962) saw no future for the phenol coefficient type of test and believed that an entirely fresh

and untrammelled approach to the problem is needed. Rogers, Maher, and Kaplan (1961) reviewed the various swabbing techniques, agar-contact methods, rinsing processes, tracer techniques, and in-use testing, and concluded, as did Walter (1955), that the method that best serves the purpose of the individual should be selected.

Testing a disinfectant on surfaces is not new; in fact, it was the first method used by Koch (1881). Different methods of using surfaces to evaluate disinfectants have been reported (Annear, 1951; Hoffman and Warshowsky, 1958; Kligman and Rosenweig, 1958; Stedman et al., 1954a, b).

In this investigation, the tissue phase of four fungi, *Blastomyces dermatitidis*, *Coccidioides immitis*, *Cryptococcus neoformans*, and *Histoplasma capsulatum*, was aerosolized on surfaces of five materials commonly used in laboratories on the work bench, floor, or equipment. Aerosolization was selected because an earlier study (Kruse, 1962) demonstrated that many laboratory mycological procedures create aerosols that may cause laboratory-acquired infection (Furcolow, 1961; Smith et al., 1961). Candidate fungicides were evaluated by concentration and time for their ability to kill the fungi on different surfaces. This study, as will a future study on cultural phases of the test fungi, attempts to approximate conditions that may occur when infectious aerosols are created by mycological laboratory techniques. The aerosolized cells or spores settle and dry on the surface. Further investigation to evaluate the action of these fungicides on the cultural phase will be reported at another time.

MATERIALS AND METHODS

Test fungi. Spherules of *C. immitis* M-11 were grown by the method of Converse (1955) and filtered by gravity through six layers of sterile surgical gauze to remove trace amounts of hyphae. The yeast cells of *H. capsulatum* 3021 were grown in 50 ml of Pine (1954) medium. *B. dermatitidis* 3110 was propagated in Brain Heart Infusion broth (Difco), and *C. neoformans* in nutrient broth (Difco) with 0.1% thiamine·HCl added. Purity and viability of the fungi were ascertained by microscopy and serial-dilution culture before and after aerosolization and drying.

Test surfaces. The following materials (1 in.²) were used in this investigation: (i) wood painted with two coats of Plicout (Fisher Scientific Co.), (ii) glass, (iii) stainless steel,

(iv) neoprene, and (v) asphalt floor tile. The surface was cleaned with detergent and water, thoroughly rinsed with distilled water, and dried. The stainless-steel and glass surfaces were sterilized by autoclaving at 121 C for 15 min. Painted wood, neoprene, and asphalt tile surfaces were sterilized with ethylene oxide.

General procedure. Oster and Golden (1949) stated that there are three basic conditions for a fungicidal test: (i) contact of the fungus and fungicide for a limited time, (ii) complete removal of the fungicide from the fungus by a suitable wash, and (iii) the basic structure of the colony unaltered by the compound or test.

In the present investigation, 1-in.² sections of test materials were placed in an aerosol chamber (Fig. 1). The test fungus was introduced into the chamber with a Vaponefrin nebulizer (Vaponefrin Co., Metuchen, N.J.) that had been modified to produce fungal-bearing particles with diameters ranging from 1 to 30 μ . After aerosolization, the residual aerosol was evacuated through a fiberglass filter. Then dry sterile air was admitted to the chamber to dry the seeded surfaces. No drying menstruum, which by its nature can be expected to exert a greater or lesser degree of

protection against attack by the disinfectant (Sykes, 1962), was used.

The test procedure is outlined in Fig. 2. The seeded surface was immersed in the disinfectant. A disinfectant should achieve its objective in as short a time as possible, and a contact time of not less than 2 nor more than 10 min should be used (Davis, 1960). Sykes (1962) regarded 5 or 10 min of contact time as too short and preferred a 30-min time with no advantage in prolonging the time beyond 30 min. We chose times of 1, 5, 10, 20, 30, and 60 min so that at a given time and concentration the disinfecting efficiency could be determined by graphic interpolation. At each of the six selected times, a sample surface was removed and immersed for 30 sec in 50 ml of an aqueous solution of a neutralizer that was specific for the test disinfectant. The entire surface sample was swabbed with a Calgiswab (Consolidated Laboratories, Inc., Chicago Heights, Ill.). Calcium alginate soluble wool was preferred to cotton, as Higgins (1950) showed that cotton did not recover as many organisms as did soluble wool. After dissolving the Calgiswab in 4 ml of 1% $\text{Na}_2\text{C}_2\text{O}_4 \cdot 2\text{H}_2\text{O}$, 0.5-ml samples of the resulting suspension were plated in triplicate on appropriate media.

To assure complete recovery of any fungus particle that may have remained on the seeded surface after swabbing, or may have washed off into the neutralizing solution, the surface was immersed in appropriate broth and the neutralizing solution was passed through a membrane filter and cultured (Orlando and Bolduan, 1953). Petri plates, tubes, and membrane filters were incubated at 37 C for 10 days. An exception was made for *H. capsulatum* because Rowley and Huber (1955) reported that on a blood medium at 27 to 30 C this organism would convert from yeast cells to mycelial colonies and better recovery would result.

A control was tested concurrently by immersing the seeded surface in sterile 0.85% saline and proceeding as with a test surface. All controls showed growth except *H. capsulatum*. Cysteine (0.1%) was incorporated in the saline solution because Rowley and Huber (1955) reported that this combination maintained the viability of *H. capsulatum* yeast cells. With this modification, *H. capsulatum* grew in the controls.

Special procedure. For use in statistical analysis, as later described, an additional set of tests was made employing *C. neoformans* (because it was the most resistant of the four organisms to fungicides), asphalt tile (because it was the most difficult to disinfect), and four disinfectants (phenol, Cresylic, Phenolic A, and Iodenic), at fungicidal concentrations of 0.4, 0.6, 1, 1.5, 2.5, 4, 6, and 10%, and at times of 0.5, 1, 1.5, 2.5, 4, 6, 10, 15, 25, 40, and 60 min.

Candidate fungicides. Fungicides tested were (i) a liquid *n*-alkyl (50% C_{12} , 30% C_{14} , 17% C_{16} , 3% C_{18}) dimethyl benzyl ammonium chloride (designated Quat.); (ii) ethyl alcohol; (iii) phenol; (iv) formaldehyde; (v) peracetic acid; (vi) sodium hypochlorite; (vii) a cresylic product (*o*-phenylphenol) containing soap and alcohol (designated Cre-



FIG. 1. Chamber used for aerosolizing fungal particles.

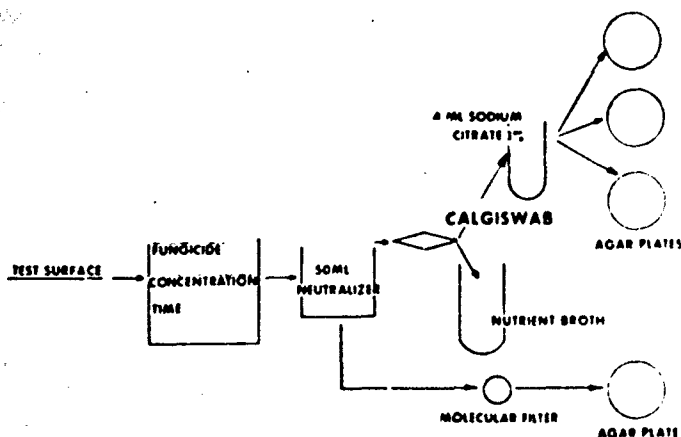


FIG. 2. Test procedure.

sylic); (viii) a phenolic formulation containing *o*-benzyl *p*-chlorophenol, *p*-tertiary amyl phenol, and *o*-phenylphenol (designated Phenolic A); (ix) a phenolic formulation containing *o*-benzyl *p*-chlorophenolate and potassium ricinoleate (designated Phenolic B); and (x) an iodophor containing polyethoxy polypropoxy ethanol-iodine complex, and nonyl phenyl ether of polyethylene glycol-iodine complex (designated Iodenic).

The disinfectants were evaluated at concentrations of 0.1, 0.5, 1, 2, 5, and 10%, with the exception of ethyl alcohol (30, 50, 70, and 90%). The disinfectant concentrations were prepared on a volumetric basis in distilled water, without regard for the specific gravity. However, Quat. (50%), formaldehyde (37%), and peracetic acid (40%) were prepared on an active-ingredient basis. The disinfectants were prepared immediately before use.

Culture media. The following media were used: (i) substrate for the membrane filter and agar for plating the resulting suspensions of the dissolved Calgiswab, and (ii) liquid broth for incubation of the swabbed surface.

For *B. dermatitidis*, the substrate and plating medium contained Brain Heart Infusion broth (Difco), 37 g; dextrose, 10 g; agar, 20 g; and distilled water, 1 liter; autoclaved at 121 C for 15 min. The broth medium was the same as the plating medium without the addition of agar.

For *C. immitis* and *C. neoformans*, the substrate and plating medium contained peptone (Difco), 10 g; dextrose, 20 g; and distilled water, 1 liter; autoclaved at 121 C for 15 min. The broth medium was the same as the plating medium without the addition of agar.

For *H. capsulatum*, the substrate and plating medium (Rowley and Huber, 1955) contained Casamino Acids (Difco), 10 g; dextrose, 20 g; calcium pantothenate, 1 mg; biotin, 0.5 mg; and distilled water, 1 liter; the pH was adjusted to 6.5; 20 g of agar were added; it was autoclaved at 121 C for 15 min and cooled to 45 C; and 60 ml of defibrinated sheep blood were added aseptically. The broth medium (Salvin, 1950) contained Casamino Acids (Difco), 10 g; dextrose, 3 g; yeast extract dialysate, 3 g; NaCl, 2.5 g; cysteine-HCl, 0.5 g; KCl, 2.5 g; Na₂HPO₄, 4 g; and distilled water to make 1 liter; autoclaved at 121 C for 20 min.

Neutralizing solution for disinfectants. The neutralizing solution for Cresylic, Phenolic A, Phenolic B, formaldehyde, and phenol consisted of the broth medium for the specific fungi (described above) plus 1% Tween 80 (polyoxyethylene sorbitan monooleate). For Iodenic, sodium hypochlorite, and peracetic acid, the specific broth media plus 0.5% (dry weight) Na₂S₂O₃·5H₂O were used. For Quat. and ethyl alcohol, the specific broth media plus 0.07% azolectin dissolved in 0.5% aqueous Tween 80 were used.

In the three solutions listed above, the specific neutralizer was added to the distilled water used to prepare broth media.

RESULTS

When tests are performed in which many factors are involved, it is essential to standardize or eliminate vari-

ables to determine the reliability of the test procedure. Because the seeded surfaces are necessarily observed at specific times, the exact time required for disinfection could not be estimated. Previous experience in statistical analysis of the effect of disinfectants on bacterial aerosols has shown that, when log transformation is applied to concentration of disinfectant, responses of the bacteria to the disinfecting process are often linearized. To test the hypothesis that the regression of "time required for disinfection" on "concentration of disinfectant" is linear in the log scale, samples of disinfectants were observed at a greater number of times and concentrations, as described under *Special procedure*. Concentrations of 0.4, 0.6, 1, 1.5, 2.5, 4, 6, and 10% were tested at 0.5, 1, 1.5, 2.5, 4, 6, 10, 15, 25, 40, and 60 min to give approximately equal intervals on the log scale for both variables. Times and concentrations were converted to logarithms, and for a given log concentration the corresponding log time was estimated by linear interpolation between two or more log times for which both positives and negatives resulted. For example, with 1.5% Cresylic disinfectant all replications resulted in negatives at 60 min; three positives and one negative occurred at 40 min. It was therefore assumed that the true time for disinfection is between 40 and 60 min. The following equation was used to determine the time required for disinfection:

$$\log t = \log \text{lower } t + \left(\frac{\text{number of positives}}{\text{total observations in } \Delta \log t} \right) \Delta \log t$$

where $\Delta \log t$ = shortest log time in which all negatives occurred minus the next shortest log time in which at least one positive was observed. A probability level of 0.01 permitted a reasonable conclusion concerning the homogeneity of slopes. It was concluded that: (i) regression for "time required for disinfection" on "concentration of disinfectant" is accurately represented as $\log Y = a + b(\log X)$, where Y = time required for disinfection, X = concentration of disinfectant, a = intercept, and, b = slope; (ii) the parameter b could be treated as constant over all disinfectant-fungus-surface combinations; and (iii) the disinfectants could be ranked according to effectiveness in terms of time with a given concentration of disinfectant. Notice (Fig. 3 through 6) that this equation does not apply to the use of ethyl alcohol. These curves were fitted by observed results rather than by any computed equation. The data presented in Fig. 3 through 6 are plotted in semilog scale and rank the fungicidal efficiency with regard to time and concentration on different laboratory surfaces.

DISCUSSION

Comparison of the four fungi. The tissue phases of *B. dermatitidis*, *C. immitis*, and *H. capsulatum* reacted comparably when subjected to a given fungicide. However, when *C. neoformans* was subjected to the same fungicide, a greater time was required for disinfection. In all probability, the capsule of *C. neoformans* impeded the penetration of the cell wall by the fungicide.

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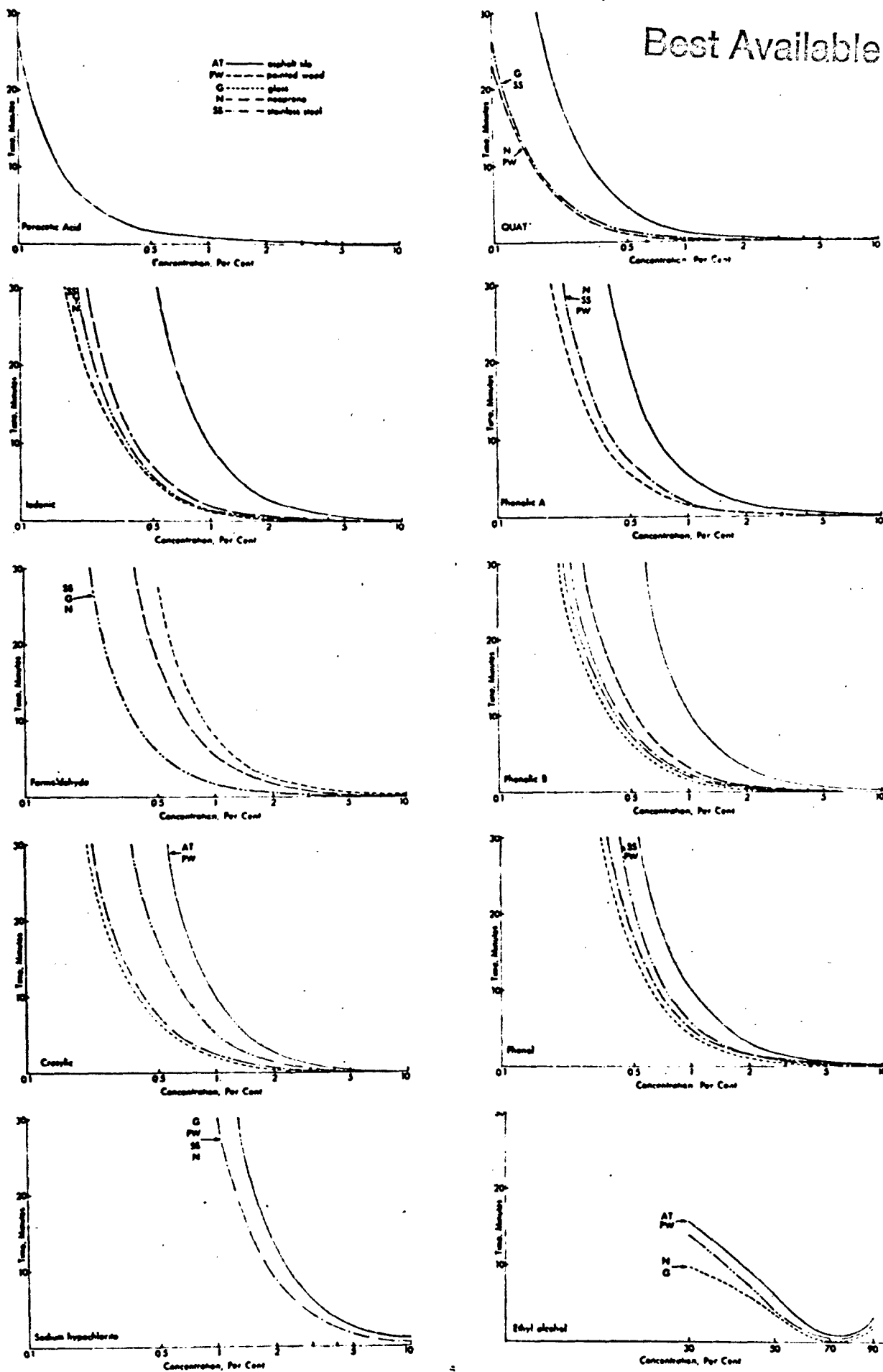


FIG. 3. Effect of fungicides on *Blastomyces dermatitidis*.

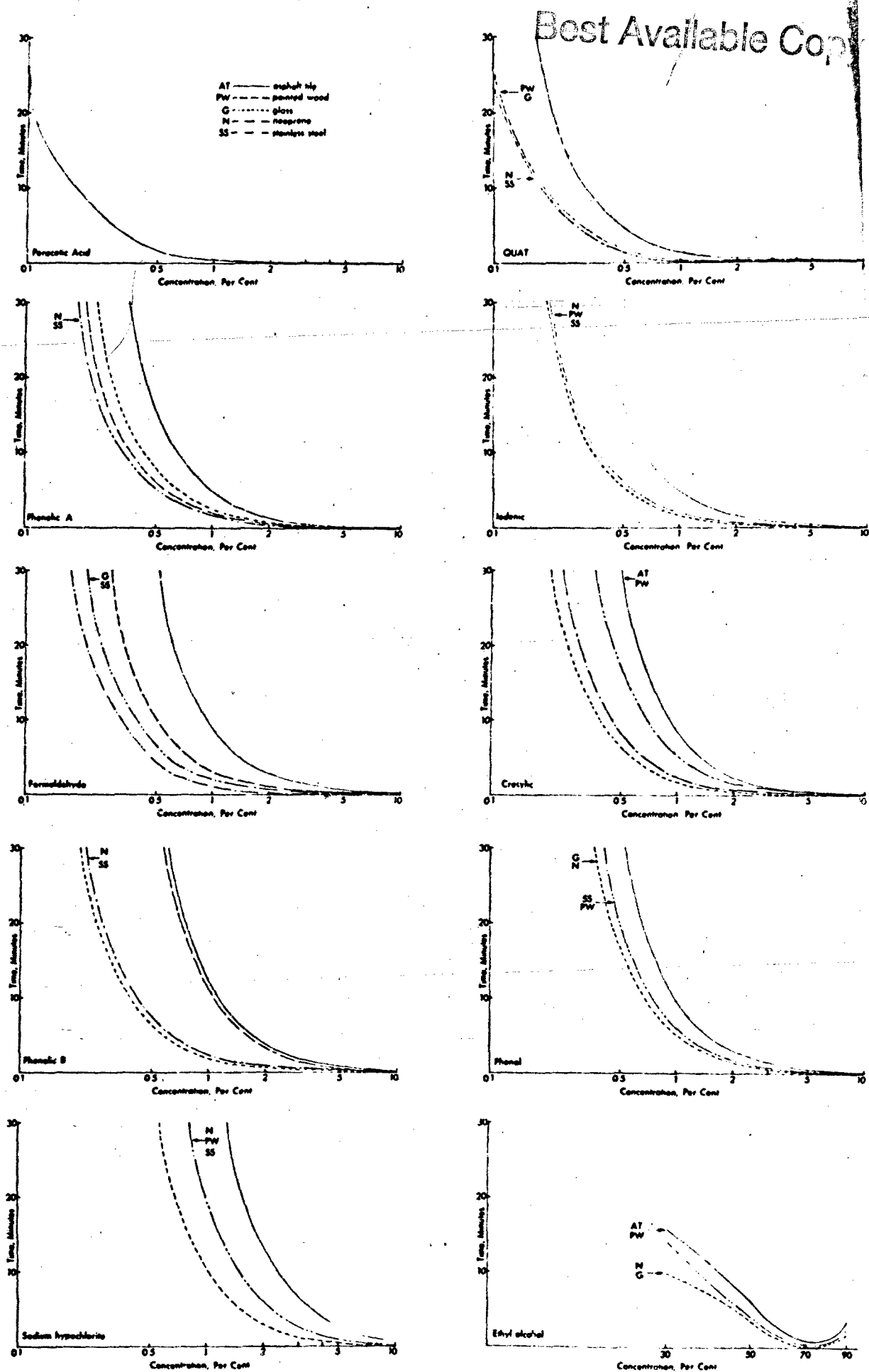


FIG. 4. Effect of fungicides on *Coccidioides immitis*.

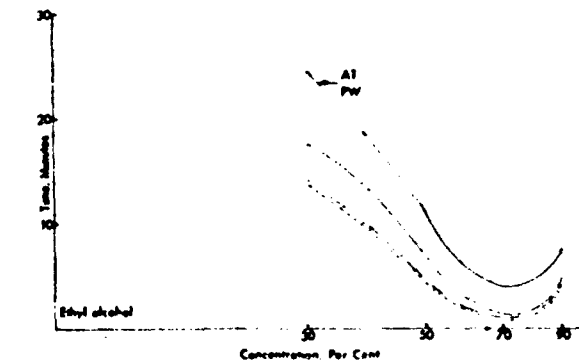
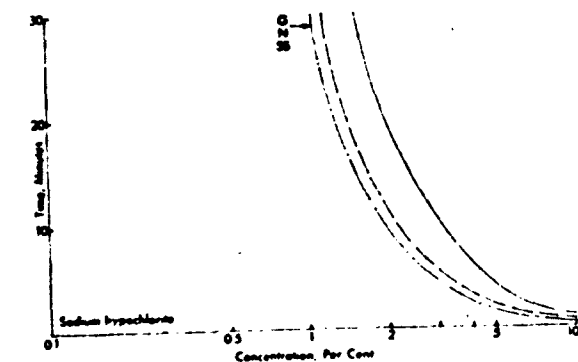
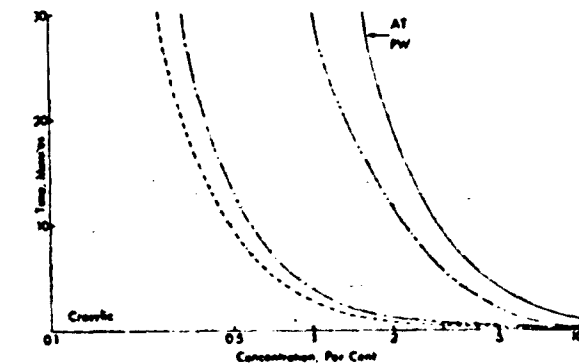
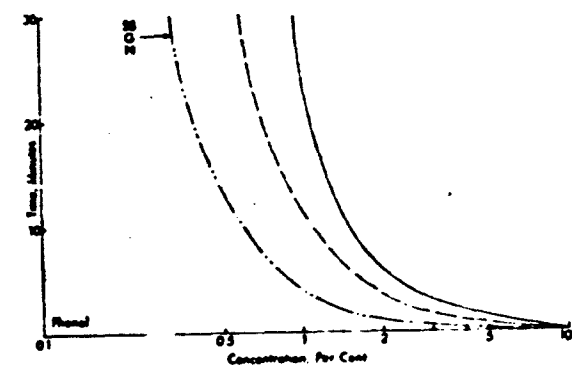
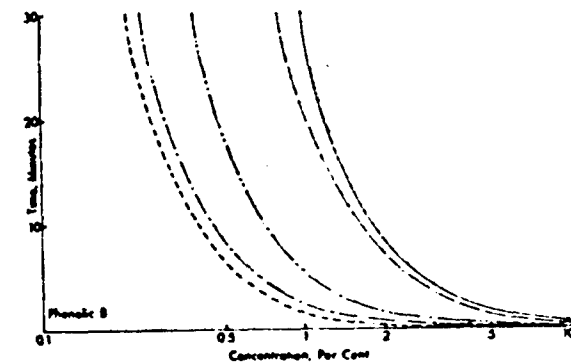
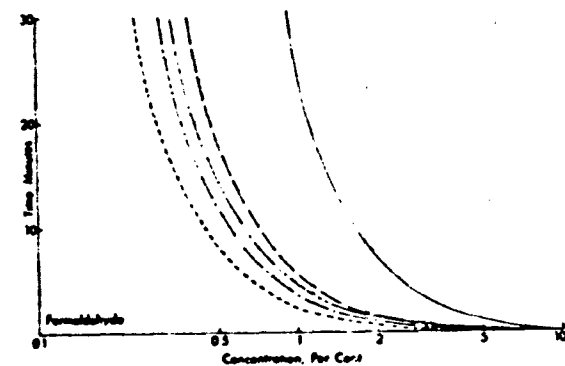
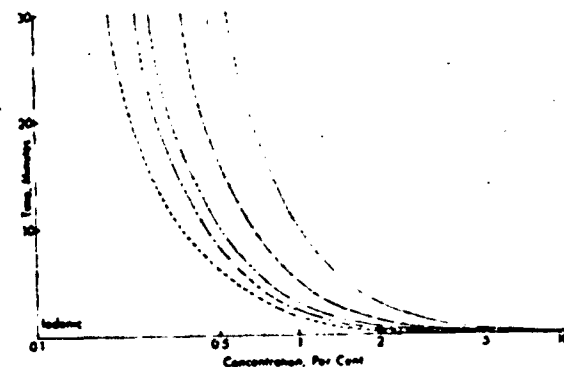
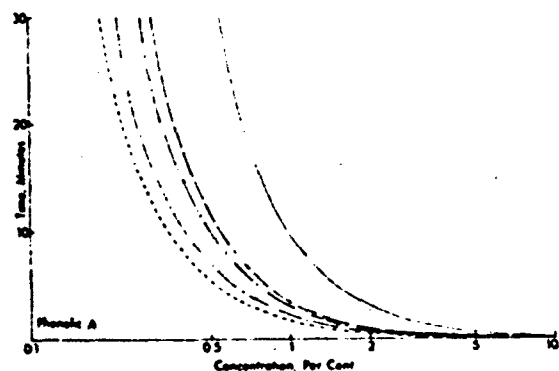
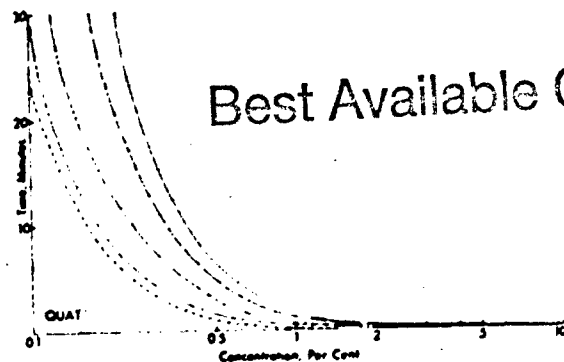
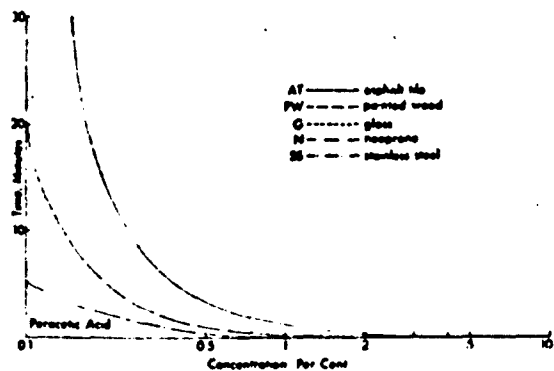


FIG. 5. Effect of fungicides on *Cryptococcus neoformans*.

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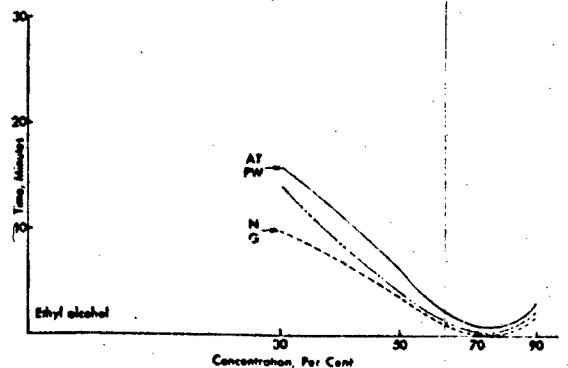
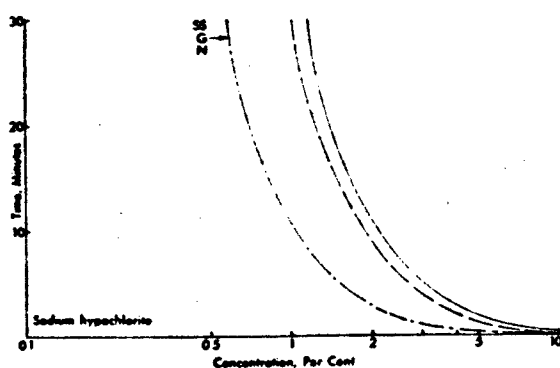
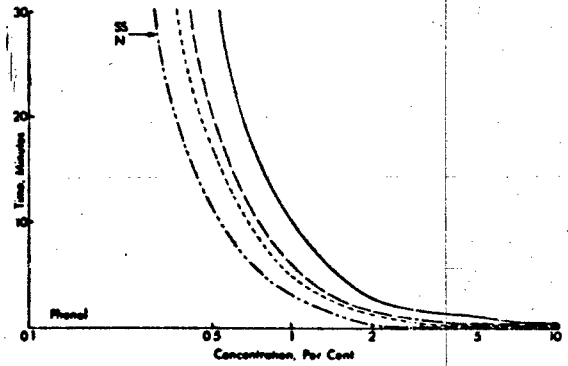
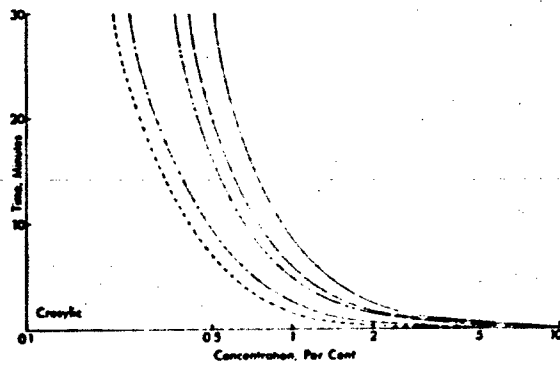
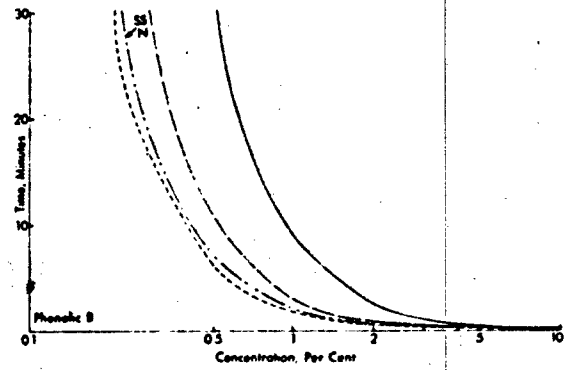
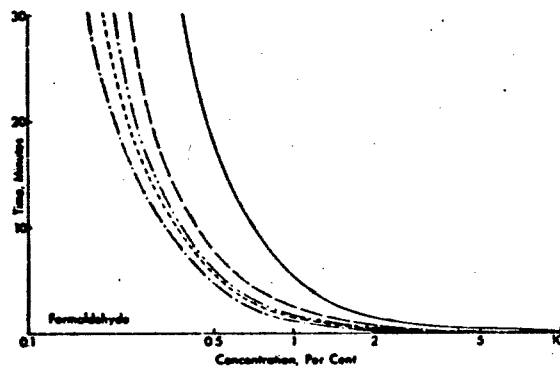
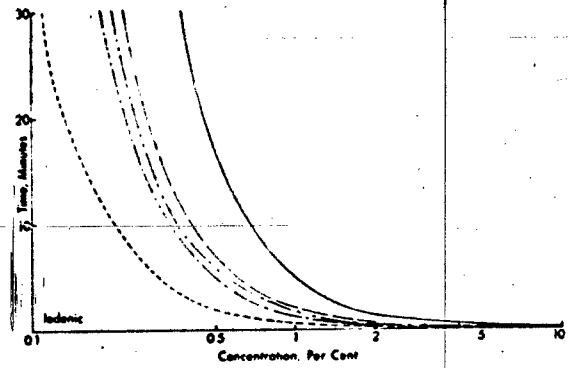
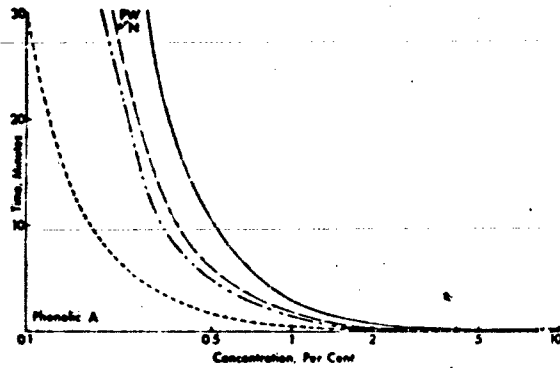
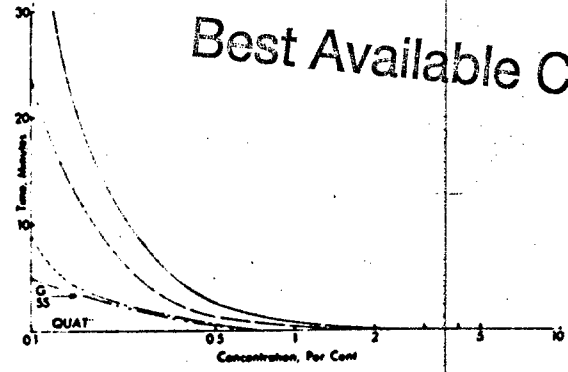
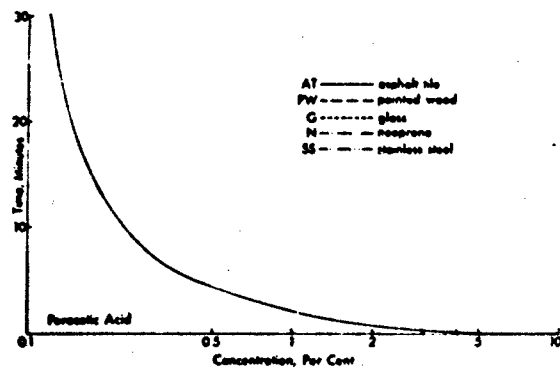


FIG. 6. Effect of fungicides on *Histoplasma capsulatum*.

Effect of the surface material. The data in Fig. 3 through 6 indicate that, when time and concentration of a fungicide are standardized, the nature of the test surface determines the fungicidal efficiency. A disinfectant will behave differently on a hard, impervious surface such as glass or metal than it will on rubber, and even more differently on a porous surface such as wood (Sykes, 1962). Since there is no method of comparing the degree of porosity of test surfaces, the surfaces were examined microscopically. It was determined that the order of decreasing porosity was: asphalt tile, painted wood, stainless steel, neoprene, and glass. The significant effect of surfaces is well known. Stedman et al. (1954b) stated that higher concentrations of disinfectants were required to disinfect porous surfaces than nonporous surfaces in a given time. They showed that phenolic, cresylic, and quaternary disinfectants were more effective against *Staphylococcus aureus* spread on stainless steel than on asphalt tile. Varley and Reddish (1936) showed that a cresylic disinfectant was more effective on *Escherichia coli* spread on glass, less so on rubber, and least effective on asphalt tile. Microorganisms were more difficult to recover from an unpainted oak surface than from stainless steel (Flannery et al., 1953). Vashkov and Nekrasova (1959) reported that painted wood surfaces were disinfected more rapidly than unpainted wood surfaces when using *S. aureus* and *E. coli*.

Correlation with pertinent reports by other investigators. Peracetic acid, quaternaries, iodenic solutions, and sodium hypochlorite are less effective when prepared on a volumetric basis (Gershenfeld and Palisi, 1949; Lawrence, Carpenter, and Naylor-Foote, 1957) than when prepared on a basis of active ingredients present (Glassman, 1948; Klarmann, 1956). Throughout this study, peracetic acid and Quat. were prepared according to the amount of active ingredients present. The hypochlorite and iodenic solutions were prepared on a volumetric basis because the amount of active ingredient varies with the manufacturer, and because in common laboratory disinfectant use the solutions are prepared by simple volumetric dilution. To permit easier comparison of the present results with those of other investigators, the amount of active ingredient for those four fungicides is presented in Table 1.

Cantor and Shelanski (1951) reported iodenic compounds to be more effective against yeast than hypochlorite and quaternaries on a basis of active ingredients present.

TABLE 1. Active ingredient in fungicidal solutions

Tested %	Iodenic	Quat	Hypochlorite	Peracetic acid
	ppm	ppm	ppm	ppm
0.1	16	500	52.5	400
0.5	80	2500	262.5	2000
1.0	160	5000	525.0	4000
2.0	320	10,000	1050.0	8000
5.0	800	25,000	2625.0	20,000
10.0	1600	50,000	5250.0	40,000

Dunn (1937) found that in the test tube 1 to 2% active-ingredient quaternary killed *C. neoformans*, *Candida albicans*, and *Saccharomyces cerevisiae* in 10 min but not in 5 min. Becco Chemical Division (1957) reported that 0.025% peracetic acid killed completely a concentration of 42×10^6 *E. coli* per ml in 1 min. In the present study, peracetic acid was found to act on the fungi so rapidly that time-concentration relationships could not be clearly plotted except with capsular *C. neoformans* on three surfaces.

Dunn (1937) showed that 1% phenol did not kill *C. albicans* or *C. neoformans* in 5 min, but that 1% was effective in 10 min. Klarmann et al. (1933) and Woodward, Kingery, and Williams (1934) reported that 1% killed *C. albicans* and *C. tropicalis* in 30 min. Vashkov, Sukhareva, and Chadova (1957) stated that compounds of phenol in which the halogen is in the *para* position to the hydroxyl group are more effective than compounds in which halogen is in the *ortho* position. Stedman et al. (1954a, b) stated that cresylic and Phenolic B disinfectants are more effective than phenol when vegetative microorganisms were dried on steel and tile surfaces. Klarmann and Shternov (1936) reported that the fungicidal efficiency for a cresylic disinfectant is approximately two times greater than phenol, and for Phenolic B is approximately four times greater. Our data in Fig. 3 through 6 indicate that Phenolic A is more effective than Cresylic or Phenolic B disinfectant.

Emmons (1933), Spaulding (1939), and Tilley (1945) reported that formaldehyde in aqueous solution is effective in 2 to 5 min against *C. albicans*.

Ethyl alcohol has been the subject of conflicting reports. With vegetative bacteria dried on threads, concentrations of 40 to 70% were germicidal but 90% was not (Harrington and Walker, 1903; Post and Nicoll, 1910). With wet surfaces, 95% ethyl alcohol was effective, but with dry surfaces 70% was better (Morton, 1950; Smith, 1951). Our data in Fig. 3-6 verify the findings of previously mentioned experiments in regard to the action of ethyl alcohol on the tissue phase of the test fungi air-dried on surfaces.

Statistical analyses of the data in Fig. 3 through 6 correlating the four fungi, five surfaces, and time and concentration indicate that heterogeneity exists among the fungicides, and ranks them in order of decreasing effectiveness as follows: (i) peracetic acid, (ii) Quat., (iii and iv) Iodenic and Phenolic A, (v) formaldehyde, (vi) Phenolic B, (vii) Cresylic, (viii) phenol, (ix) sodium hypochlorite, and (x) ethyl alcohol. However, with the exception of the rapid activity of peracetic acid and Quat., and the greatly reduced activity of sodium hypochlorite and ethyl alcohol, the fungicidal efficiency of the remaining fungicides is approximately equal and they can be substituted for each other. All the fungicides at a proper time and concentration are effective. Laboratory personnel using data in Fig. 3 through 6 can, by interpolation, determine, with a given fungicide, what time and concentration are required to disinfect instruments, pipettes, gloves, and, in case of a laboratory accident, bench tops and floors, when myco-

logical procedures involve the tissue phase of *Blastomyces*, *Coccidioides*, *Cryptococcus*, and *Histoplasma*.

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